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Differential Inactivation of Alcohol Dehydrogenase Isoenzymes in *Zymomonas mobilis* by Oxygen

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***Zymomonas mobilis* is endowed with two isoenzymes of fermentative alcohol dehydrogenase, a zinc-containing enzyme (ADH I) and an iron-containing enzyme (ADH II). The activity of ADH I remains fully conserved, while ADH II activity decays when anaerobic cultures are shifted to aerobiosis. This differential response depends on the metal present on each isoenzyme, since pure preparations of ADH I are resistant to oxidative inactivation and preparations of zinc-containing ADH II, obtained by incubation of pure ADH II with $ZnCl_2$, showed no modification of the target for oxidative damage (His^{277}-containing peptide). It was consistently found that the activity of the zinc-containing ADH II, once submitted to oxidative treatment, was fully restored when iron was reintroduced into the enzyme structure. These results indicate that zinc bound to these proteins plays an important role in the protection of their active centers against oxidative damage and may have relevant biochemical and physiological consequences in this species.**

Alcohol dehydrogenase II (ADH II) of *Zymomonas mobilis* belongs to the microbial iron-activated group III of dehydrogenases (7, 15), and it is the dominant enzyme during fermentation of sugars in rich medium, converting acetaldehyde to ethanol by using NADH as a coenzyme (11, 14). In a previous report (5), we demonstrated that ADH II was irreversibly inactivated by metal-catalyzed oxidation (MCO) in a process that affected the His^{277}-containing peptide. This target for oxidative damage was similar to that described for propanediol oxidoreductase of *Escherichia coli*, another enzyme of the type III group of dehydrogenases, that we proved to be irreversibly inactivated by MCO (6). The MCO of proteins is a site-specific mechanism, as extensively reviewed by Stadtman (16).

ADH I of *Z. mobilis* is an isoenzyme of ADH II that is dominant during fermentation of sugars in minimal medium. In contrast to ADH II, ADH I is a zinc dehydrogenase that belongs to the microbial group I NAD(P)-dependent ADHs (15). The amino acid sequence of this enzyme shows 30% similarity to those of eukaryotic zinc-containing long-chain ADHs (9, 10).

It has been suggested that the antioxidant properties of zinc derive from its ability to compete with prooxidant metals for binding sites, decreasing their ability to promote MCO in a particular environment (4, 19). In this context, we present experiments showing that under aerobic conditions, ADH I is resistant and ADH II is sensitive to oxidation, and this differential response depends on the metal present in the active center of each isoenzyme.

MATERIALS AND METHODS

Organisms, growth conditions, and enzyme activities. The strain used was *Z. mobilis* (ATCC 29191). Cells were grown in rich medium for ADH II purification and in minimal medium for ADH I purification. The compositions of the media used were described by Mackenzie et al. (13). Enzyme activities were measured as ethanol or butanol oxidation as described by Neale et al. (14).

Protein purification methods. ADH II was purified as described by Cabiscol et al. (5). ADH I was purified according to the following protocol. Cells were harvested by centrifugation and resuspended in four times their wet weight in 20 mM MES (morpholineethanesulfonic acid)-KOH buffer (pH 6.5) supplemented with 50 μM $ZnCl_2$. The crude extract was obtained by sonic disruption and followed by centrifugation at 15,000 × *g* for 30 min. This preparation was applied to a gel filtration column (Sephacryl S200 [Pharmacia]) and equilibrated and eluted with the zinc-containing MES buffer. Fractions with enzyme activity were pooled and applied to an ionic-exchange column (DEAE 15 HR [Waters]). Proteins were eluted with a linear gradient (0 to 0.5 M NaCl in MES buffer in 50 min) at a 5-ml/min flow rate. Fractions with activity were pooled, applied directly to a NAD-agarose affinity column (Sigma; N-1008), equilibrated and washed with MES buffer, and eluted with 2 mM NAD in the same buffer. This step yielded a preparation of ADH I that appeared as a single band in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The specific activity was 150 U/mg. Pure preparations of ADH I and ADH II were dialyzed against MES-KOH buffer (pH 6.5). Protein was determined by the Bradford procedure (3).

In vivo enzyme inactivation. Cells grown at 30°C in complex medium in the absence of oxygen to an optical density at 600 nm of 3 were harvested by centrifugation, washed once with fresh complex medium without glucose, resuspended to an optical density of 0.3 in the same medium plus chloramphenicol (10 μg/ml), and incubated in a rotary shaker (250 rpm). At the indicated times, samples were taken to determine enzyme activities.

In vitro enzyme inactivation. For in vitro enzyme inactivation, 1 to 3 nmol of purified enzyme was oxidized by incubation in conical glass vials, containing 50 μM ammonium ferrous sulfate and 100 μM H_2O_2. Vials were flushed with nitrogen for 5 min before the enzyme addition (6).

Carbonyl group determination. The carbonyl content of the native and oxidized proteins was measured by reaction with 2,4-dinitrophenylhydrazine in SDS followed by high-performance liquid chromatography (HPLC) gel filtration with a Shodex KW-803 column, according to the method described by Levine et al. (12). The estimated extinction coefficients were 21,000 M^{-1} cm^{-1} for ADH I and 22,000 M^{-1} cm^{-1} for ADH II.

Peptide mapping. Preparations of purified ADH II (300 μg) were digested with subtilisin as described by Cabiscol et al. (5).

Metal analyses. Iron and zinc were measured by inductively coupled plasma optical emission spectrometry with a Jobin-Ivon 38 spectrometer. Samples were submitted to HPLC gel filtration in a Protein Pak 125 (Waters) prior to metal analysis in order to eliminate reagents and metals not bound to the enzyme. The eluent used was MilliQ water (resistivity greater than 18 MΩ). Fractions were collected in metal-free polypropylene tubes.

RESULTS AND DISCUSSION

In *Z. mobilis*, ADH II and I are isoenzymes, which use iron and zinc, respectively, and which catalyze the conversion of acetaldehyde to ethanol with NADH as a coenzyme. To observe the effect of an aerobic environment on the activity of these enzymes, a cell culture was shifted from anaerobic to aerobic conditions. Figure 1 shows that after 4 h of incubation, ADH II showed an 80% inactivation, while ADH I remained fully active. This loss was not due to a degradation of ADH II

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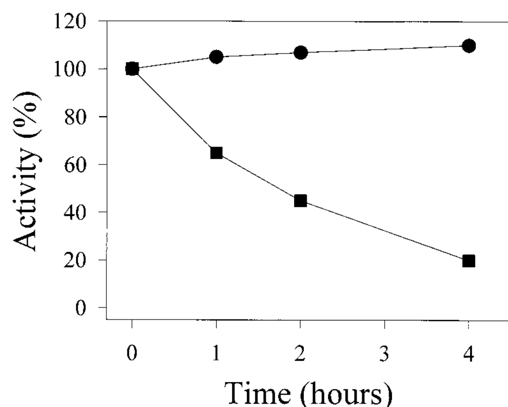

FIG. 1. In vivo inactivation of ADH I and ADH II by oxygen. Cells grown anaerobically were shifted to aerobic conditions (time zero), and ADH I (●) and ADH II (■) activities were measured at different times. The specific activities were set to 100% at time zero. The specific activities were 0.1 U/mg for ADH I and 3.0 U/mg for ADH II.

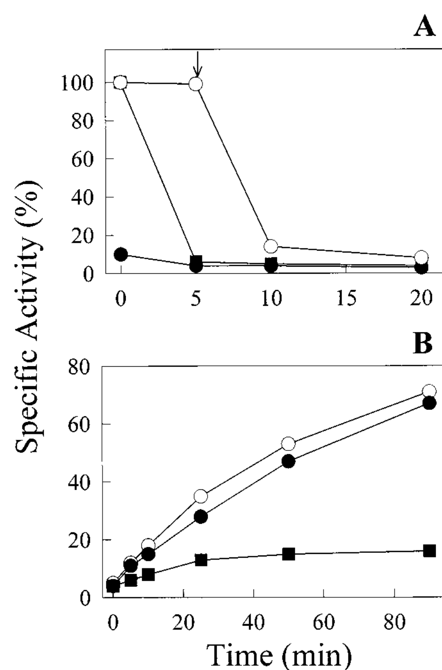

FIG. 2. Recovery of ADH II activity after MCO treatment. Native ADH II (■) and zinc-treated ADH II (●) were incubated with H_2O_2/Fe^{2+} as described in Materials and Methods, and the effect on activity was measured. An aliquot of native ADH II was kept as a control without treatment (○). After 5 min, 10 mM *o*-phenanthroline was added to each sample (indicated by the arrow), and the effect on activity was measured (A). After gel filtration, samples were incubated with 100 μM ammonium ferrous sulfate, and the activity was measured over a 90-min period (B).

protein as measured by SDS-polyacrylamide gel electrophoresis (not shown). These results can be explained by the fact that ADH II contains iron, a prooxidant metal, which after the shift to aerobic conditions will lead to oxidative inactivation of the enzyme. In contrast, ADH I was protected from inactivation because it contains zinc in the active center. In an obligately fermentative bacterium such as *Z. mobilis*, the persistence of ADH I activity could be seen as a safeguard for the restoration of full ethanologenic capacity required under favorable nutritional conditions.

This proposal was corroborated by in vitro studies with purified preparations of both enzymes exposed to an MCO system. After 2 min of treatment with H_2O_2/Fe^{2+}, ADH I retained 70% of the initial activity, in contrast to ADH II, which was completely inactivated. When iron was not added to the system, ADH I remained fully protected while ADH II was 80% inactivated (due to the iron present on the active center). These results agree with carbonyl content of native ADH II (0.09 mol of 2,4-dinitrophenylhydrazine/mol of protein subunit) and that of the oxidized enzyme (0.51 mol/mol). In contrast, MCO-treated ADH I gave a value of 0.12 mol/mol, which is close to control conditions (0.04 mol/mol). Since iron did not replace zinc during oxidative treatment of ADH I (data not shown), the observed 30% decrease in its activity would be explained by unspecific protein damage that can affect the catalytic activity.

To further investigate whether zinc present in the active center of a dehydrogenase can act as an enzyme protector against oxidative inactivation, samples of the purified ADH II enzyme (containing 0.6 atom of iron and 0.2 atom of zinc per subunit) were incubated at 30°C with 50 μM $ZnCl_2$ for 90 min. At the end of the incubation period, iron was almost completely displaced by zinc, since the metal content was 0.1 atom of iron and 0.8 atom of zinc per subunit. As a consequence of iron replacement, a 90% decrease in total activity was observed. The 0.2 atom of zinc per subunit found in pure preparations of native ADH II would be explained by the presence of zinc (6 μM) and iron (11 μM) in the complex media used (13) and the higher value of the apparent dissociation constant of ADH II for zinc compared to that for iron (18).

Zinc-containing ADH II, described in the previous section, and native Fe-ADH II were submitted to MCO. In both cases, the remaining activity after 4 min of treatment was 5% of the activity values of the untreated enzyme used as a control (Fig. 2A). At the time indicated by the arrow the three samples were incubated for 20 min with *o*-phenanthroline to remove metal ions from the enzymes. This treatment caused inactivation of the control enzyme due to iron removal. After gel filtration on a Protein Pak 125 column to eliminate the reactants used, the three samples were then incubated with MES-KOH buffer containing 100 μM ammonium ferrous sulfate, and their specific activity was determined (Fig. 2B). The activities of the zinc-containing ADH II and the control enzyme were restored to 70% of the initial value after 90 min; only 10% of the activity of the oxidized enzyme was recovered. The carbonyl content obtained after MCO treatment in the zinc-containing ADH II was 0.14 mol of 2,4-dinitrophenylhydrazine/mol of subunit, close to the value for the control enzyme (0.09 mol/mol). These data indicated that the zinc bound to ADH II shielded the active center from active oxygen species generated by H_2O_2/Fe^{2+} present in the incubation media and consistently allowed the recovery of enzyme activity after reintroduction of iron.

The His^{277}-containing peptide of a subtilisin digest had been identified as a target sequence for oxidative damage in ADH II (5). Under our chromatographic conditions, this peptide, indicated by an arrowhead, eluted with a retention time of 31.9 min in the native enzyme (Fig. 3A) and was not present in the peptide map of the oxidized enzyme (Fig. 3B). In contrast, MCO-treated zinc-bound enzyme (Fig. 3C) showed the presence of the His-containing peptide, thus indicating that the target peptide was not affected by oxidation when zinc was bound to the enzyme. No other repetitive difference was observed.

The results obtained strongly suggest that the iron binding site of ADH II would be occupied by zinc, thus protecting it

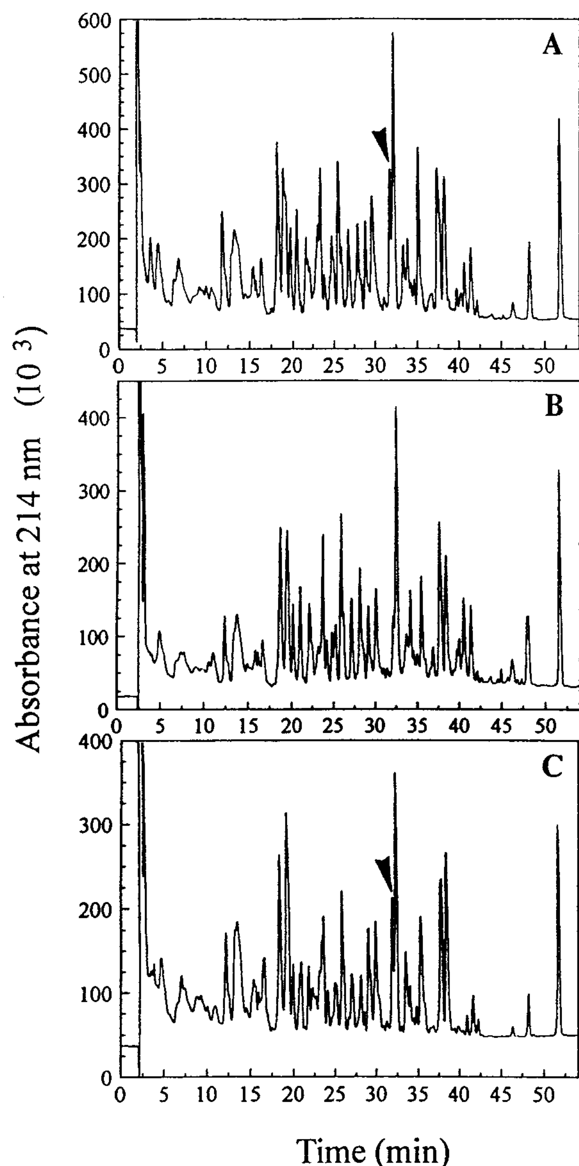

FIG. 3. Reverse-phase HPLC profiles of ADH II digested with subtilisin. Results are shown for native (A), MCO-treated (B), and MCO-treated, zinc-bound (C) ADH II. The conditions for digestion and separation are referred to in Materials and Methods. The arrowheads indicate the presence of the His-containing peptide.

against oxidation. In ADH II, the motif His^{263}-X_3-His^{267} was proposed as an iron binding motif, lying 10 residues from the MCO target. Similar motifs have been described for other iron-containing enzymes (17) and for zinc-containing enzymes (1, 2, 8). If the motif described in ADH II could be modified to use zinc instead of iron as the catalytic metal, the Fe-dehydrogenases of organisms adapting to aerobic environments would have, in terms of enzyme viability, a convenient evolutionary pathway to render their active centers more resistant to reactive oxygen species.

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